BOUNDARY MODE FRICTIONAL PROPERTIES OF ENGINEERED CARTILAGINOUS TISSUES

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Abstract

Despite the fact that lubrication is a primary function of articular cartilage, there is little information on the frictional properties of cartilaginous engineered tissues. A biochemical mediator of cartilage frictional properties in boundary lubrication, lubricin, has been shown to be secreted from chondrocyte-hydrogel constructs. In the current studies we utilized articular chondrocytes (CON), meniscal fibrochondrocytes (MEN), and mesenchymal stem cells (MSC) in alginate cultures to determine lubricin localization and the inherent boundary lubrication friction coefficient. Additionally, we investigated the ability of these tissues to be lubricated by synovial fluid and the reversibility of this lubrication. Cell-alginate constructs were cultured over six weeks, culture medium assayed for lubricin release by ELISA and constructs analyzed with immuno-histochemical (IHC) methods to investigate the localization of lubricin. Engineered tissues were tested in a custom friction instrument to determine the equilibrium friction coefficient (μ_{eq}) in boundary lubrication mode, following incubation with equine synovial fluid (SF), and subsequent extraction in l.5M NaCl. MSCs released 10 fold more lubricin than CON or MEN cultures. IHC analysis showed no localization of lubricin to alginate, minimal focal staining of engineered constructs at six weeks in culture, and the ability of all engineered tissues to localize lubricin when exogenously treated with SF. Frictional characterization showed no difference in μ_{eq} over culture for all engineered tissues, while incubation in SF decreased μ_{eq} for all tissues over culture duration, and extraction of lubricin resulted in a loss of lubrication of all engineered tissues.

Key Words: friction, lubricin, PRG4, boundary lubrication, tissue engineering, chondrocytes, meniscal fibrochondrocytes, mesenchymal stem cells

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Introduction

Articular cartilage, an avascular, aneural tissue found at the ends of articulating bones, is responsible for providing a load-bearing, low friction interface for diarthrodial joints. In joints, the primary source of lubricant is synovial fluid, which contains a variety of macromolecules synthesized by joint tissues. Components of synovial fluid contribute to joint lubrication across a variety of mechanical mechanisms from hydrodynamic to boundary modes. Boundary lubrication occurs through molecules localized at the tissue surface under conditions of high compressive loads and low entraining speeds. Under such conditions the asperities of the two cartilage surfaces interact and surface chemistry dominates the lubrication properties. This interaction produces the highest friction coefficients (μ) of any mode of lubrication (Charnley, 1960; Wright and Dowson, 1976; Roberts *et al.*, 1982), and thus the maximum potential to cause wear and transfer high shear stresses to the articular cartilage. While the structure of cartilage in part accomplishes the low friction function of cartilage (Krishnan *et al.*, 2004), biochemical interactions at the surface of the tissue have been shown to reduce the μ under boundary mode conditions (Schmidt and Sah, 2006).

One such biochemical mediator of frictional properties is lubricin, a glycoprotein found to lubricate load bearing surfaces that localize the molecule, including articular cartilage (Schumacher *et al.*, 1994), meniscus (Schumacher *et al.*, 2005; Sun *et al.*, 2006), and tendon (Rees *et al.*, 2002; Rhee *et al.*, 2005; Sun *et al.*, 2006). Lubricin, also referred to as proteoglycan 4 (PRG4) (Ikegawa *et al.*, 2000), megakaryocyte stimulating factor (MSF) precursor (Flannery *et al.*, 1999; Jay *et al.*, 2000), superficial zone protein (SZP) (Schumacher *et al.*, 1994) and camptodactyly-arthropathy-coxa vara- pericarditis (CACP) protein (Marcelino *et al.*, 1999) is found in synovial fluid (Swann *et al.*, 1981) and is secreted by superficial zone chondrocytes (Schumacher *et al*, 1994; Klein *et al.*, 2003), select cells in the meniscus (Schumacher *et al.*, 2005) and synoviocytes (Schumacher *et al.*, 1999; Jay *et al.*, 2001). Localization of lubricin in the tissue is limited primarily to the surface of the tissues lining the joint with little accumulation within joint tissue extracellular matrix (Schmidt *et al.*, 2004; Schumacher *et al.*, 2005). Previous studies have demonstrated that lubricin is removed from the surface of articular cartilage by extraction with 1.5M NaCl (Jones *et al.*, 2007), indicating that tissue localization in cartilage is not the result of covalent attachment. The efficacy of lubricin in boundary mode has been shown in material-material

interfaces including latex-glass (Jay, 1992; Jay and Hong, 1992; Jay *et al.*, 1998), in cartilage-cartilage interfaces (Schmidt and Sah, 2006), and in preliminary studies of cartilage-glass interfaces (Gleghorn *et al.*, 2006b). While boundary lubrication in a dose dependant manner is evident by purified lubricin (Schmidt and Sah, 2006), the specific mechanism is unknown.

Efforts to regenerate or engineer joint tissues have typically focused on producing tissues with proper compressive or tensile properties (LeBaron and Athanasiou, 2000; Sweigart and Athanasiou, 2001; Waldman *et al.*, 2003; Hung *et al.*, 2004; Seidel *et al.*, 2004; Klein *et al.*, 2007). Despite the fact that lubrication is a primary function of articular cartilage, there is little documentation of efforts to engineer tissues with proper frictional properties. Current *in vitro* efforts with engineered cartilage focus on evaluating the expression (Grad *et al.*, 2005; Grad *et al.*, 2006) and secretion (Klein *et al.*, 2003; Schmidt *et al.*, 2004; Grad *et al.*, 2005; Klein *et al.*, 2006) of lubricin from chondrocytes under various culture conditions and medium supplements. These studies have focused primarily on production of lubricin by articular chondrocytes, but little is known about the ability of other types of chondrocytes, such as meniscal fibrochondrocytes, to generate lubricin in 3D culture. Further, there has been great interest in the use of mesenchymal stem cells (MSCs) as a cell source for cartilage tissue engineering, but little is known about the ability of these cells to generate lubricin after chondrogenic differentiation. Regardless of cell source, there is little information on the frictional properties of cartilaginous engineered tissues.

Toward this end, the objectives of this study were to utilize three distinct cell types, articular chondrocytes, meniscal fibrochondrocytes, and bone marrow derived mesenchymal stem cells in three dimensional alginate cultures to determine: 1) the production and localization of lubricin; 2) the inherent boundary lubricated friction coefficient of these engineered tissues; 3) the ability of these tissues to be lubricated by lubricin from synovial fluid; and 4) the reversibility of synovial fluid lubrication by removal of lubricin via extraction with salt solutions.

Methods

Cell / lubricant preparation
Equine sternal bone marrow mesenchymal stem cells (MSC) and stifle joint synovial fluid (equine synovial fluid, ESF) were aspirated from three geldings (22-24 months old) within 15 minutes of euthanasia following protocols approved by the Cornell University Institutional Animal Care and Use Committee. Bone marrow aspirates were pooled and plated using standard protocols (Worster *et al.*, 2000) to isolate MSCs. Briefly, aspirates were washed and plated in tissue culture flasks with growth medium in an incubator at 37°C and 5% CO_2 environment, resulting in a population of adherent cells, taken to be MSCs, from the bone marrow aspirate after 24 hours. The medium was changed, removing non-adherent cells, and the flasks were washed with sterile phosphate buffered saline (PBS)

(Mediatech, Herndon, VA), prior to the removal of MSCs from the culture flasks with trypsin (Sigma, St. Louis, MO) for 4 minutes. ESF aspirates were visually inspected to ensure blood and contaminate free, pooled, and frozen at -20°C until use in friction testing.

Primary bovine chondrocytes (CON) and meniscal fibrochondrocytes (MEN) were sterilely isolated from harvested patellofemoral groove cartilage and both lateral and medial menisci from four 1-3 day old calves. The tissue was pooled and underwent similar cell isolation protocols by digestion for 18 hours with 0.3% collagenase digest followed by a series of washing and centrifugation steps (Genes *et al.*, 2004; Ballyns *et al.*, 2005). All tissues collected were from animals with no musculoskeletal pathologies.

Creation of engineered tissues
Cell-alginate constructs were created at a seeding density of 2.5×10^7 cells/mL and cultured in an incubator at 37°C and 5% CO_2 environment for 0, 2, 4, and 6 weeks. MSC, CON, and MEN were encapsulated in 20 mg/mL of Protanal LF 10/60, a low viscosity alginate with a mean guluronate/mannuronate ratio of 70/30 (FMC Biopolymer, Drammen, Norway) in PBS. Cell-alginate suspensions were mixed with 20 mg/mL calcium sulphate (Mallinckrodt Baker, Phillipsburg, NJ) in PBS in a 2:1 ratio via a 3-way stopcock (Chang *et al.*, 2001). The resulting mixture was injected between two parallel plates separated by a 1mm spacer to produce a sheet of cell-seeded gel. From this sheet, 6 mm disks were cut using a dermal biopsy punch, resulting in 48 disks (12 disks/time point) for each of the cell-alginate conditions. The disks were placed in culture with standard culture media supplemented with 10% fetal bovine serum (FBS) (Gemi Bio-Products, Woodland, CA), 100 U/mL penicillin (Mediatech) and 100 μg/mL streptomycin (Mediatech). In addition, MSC media was supplemented with 5 ng/mL TGF-β1 (Peprotech, Rocky Hill NJ) to enhance chondrogenic differentiation (Worster *et al.*, 2000; Worster *et al.*, 2001). For all conditions, media was changed every three days, with spent media stored at -20°C for biochemical analysis.

Following culture at the respective time points, samples were stored for subsequent mechanical and biochemical characterization. Cell-alginate constructs for confined compression testing followed by biochemical characterization (n=5) and friction testing (n=5) were removed from culture and frozen at -20°C for later testing. Histological cell-alginate samples (n=2) were removed from culture and placed in 10% neutral buffered formalin (Fisher Diagnostics, Middletown, VA) supplemented with 1mM $CaCl_2$ (Sigma) to prevent gel dissolution (Nakamura *et al.*, 1997). Acellular controls were created utilizing the same protocols for cellular constructs and were frozen at -20°C for later confined compression (n=5) and friction testing (n=5).

Characterization of engineered tissues
Cell-alginate samples and acellular controls for mechanical testing were thawed in PBS with an EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim,

Germany) to prevent dissolution of the alginate gel due to calcium chelation by EDTA. The protease inhibitor cocktail focuses on inhibiting serine and cysteine proteases while the matrix metalloproteinase (MMP) protease activity was limited by minimizing the time from thawing to mechanical testing. Samples were thawed, equilibrated in PBS, and mechanically tested within 1 hour.

Confined compression testing (n=5 samples/time point/ cell type, n=5 acellular controls) was performed for comparison of compressive properties to values obtained in the literature for CON-, MEN-, and MSC-alginate generated tissue. Cell-alginate constructs were tested using an ELF-3200 test system (Bose EnduraTEC, Minnetonka, MN) in confined compression with the application of 50 µm displacement steps applying 5-45% strain. Each displacement step was held for 12 minutes which was determined as a minimum of three times the time constant for 6 week constructs (τ_{CON}=2.8, τ_{MEN}=2.7, τ_{MSC}=2.3 minutes) from preliminary data (not shown) ensuring full relaxation of the sample. The resulting compressive loads were fit to a poroelastic model (Quinn and Grodzinsky, 1993) allowing for the calculation of an equilibrium modulus (H_A) and permeability (k) from the equilibrium stress-strain curve (Figure 2c inset figure, example 6 week data).

Immediately following confined compression testing the samples were papain (Sigma, St. Louis, MO) digested at 60°C for 14 hours and standard biochemical assays were utilized for biochemical characterization of proteoglycan and collagen localization. Glycosoaminoglycan (GAG) content was assayed with a modified dimethyl methylene blue (DMMB) dye assay (Enobakhare *et al.*, 1996) at low pH, and a dimethylaminobenzaldehyde (DMAB) assay (Neuman and Logan, 1950) was utilized to measure the hydroxyproline content of the engineered tissues.

Analysis of lubricin synthesis and localization
Media from the CON, MEN, and MSC constructs was thawed and assayed to determine quantities of lubricin released to the media from the cell-alginate constructs over culture time (n=10 samples/cell type). A direct ELISA using rabbit polyclonal antibody 06A10 (Young *et al.*, 2006) was developed to assay the lubricin concentration in the conditioned media from the respective samples, using recombinant human lubricin as a standard.

Following fixation in formalin, the tissue was embedded and sectioned into 5 µm thick sections using standard histological procedures to investigate tissue localization of lubricin. Sections underwent immunohistochemical (IHC) staining for lubricin using monoclonal antibody 3-A-4 as previously described (Schumacher *et al.*, 1999). To assess the ability of lubricin to bind to engineered tissues, sections were incubated with bovine synovial fluid (BSF) for one hour at 20°C. BSF treated sections were washed twice with tris buffered saline (TBS)/0.1% Tween at 20°C and once with TBS at 20°C before IHC staining for lubricin.

Friction testing
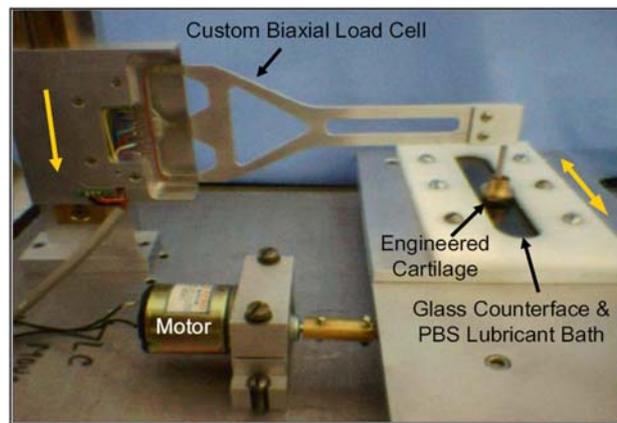The engineered tissue and acellular controls were tested

Figure 1: Picture of the custom friction instrument consisting of a custom biaxial load cell, a glass counterface, PBS lubricant bath, and servo motor which moves a linear slide. The biaxial load cell applies a normal strain to the tissue sample as the table moves a glass counterface parallel to the sample surface to create a frictional interface. The gold arrows indicate translation of the load cell (left) and linear slide (right). The load cell simultaneously measures the applied normal and resulting frictional shear loads allowing for the calculation of the friction coefficient (µ).

in a custom friction apparatus (Gleghorn *et al.*, 2006b) using PBS as a lubricant (Figure 1). Briefly, the friction apparatus linearly oscillates each sample against a glass counterface (root mean square (RMS) roughness 5nm ± 0.17nm) at a controlled speed with imposed normal strains on the tissue. A servo motor driven linear slide oscillates glass under a tissue sample while a custom biaxial load cell [normal direction: 2.5 kg max load, resolution = 1 g; shear direction: 250 g max load, resolution = 10 mg] applies a normal strain and simultaneously measures the resulting normal and frictional shear loads on the sample. The tissue samples are submerged in a lubricant bath of PBS throughout the duration of testing. Custom Matlab code (The Mathworks, Natick, MA) is utilized to calculate the equilibrium frictional coefficient (μ_{eq}), which is the ratio of the normal load to the shear load when the engineered sample has fully relaxed from the applied normal strain.

Prior to testing, a Stribeck curve (Hersey, 1938) was created to determine appropriate entraining speeds and normal strains to produce boundary mode lubrication for engineered samples lubricated with PBS (data not shown). In this Stribeck analysis, μ_{eq} was measured over a range of entraining speeds from 0.25 mm/sec to 5 mm/sec and normal strains from 10% to 50%. The region of the speed-strain space that yielded constant μ_{eq} was considered boundary lubrication. As such, for these boundary lubrication studies, the instrument linearly oscillated each sample at 0.32 mm/sec against glass with an imposed normal strain of 30% for 40 minutes to allow full relaxation of the sample. The application of 30% normal strain results in an approximate normal stress of 3, 8, and 10 kPa for MSC, CON, and MEN respectively. PBS was utilized as a lubricant for all friction tests.
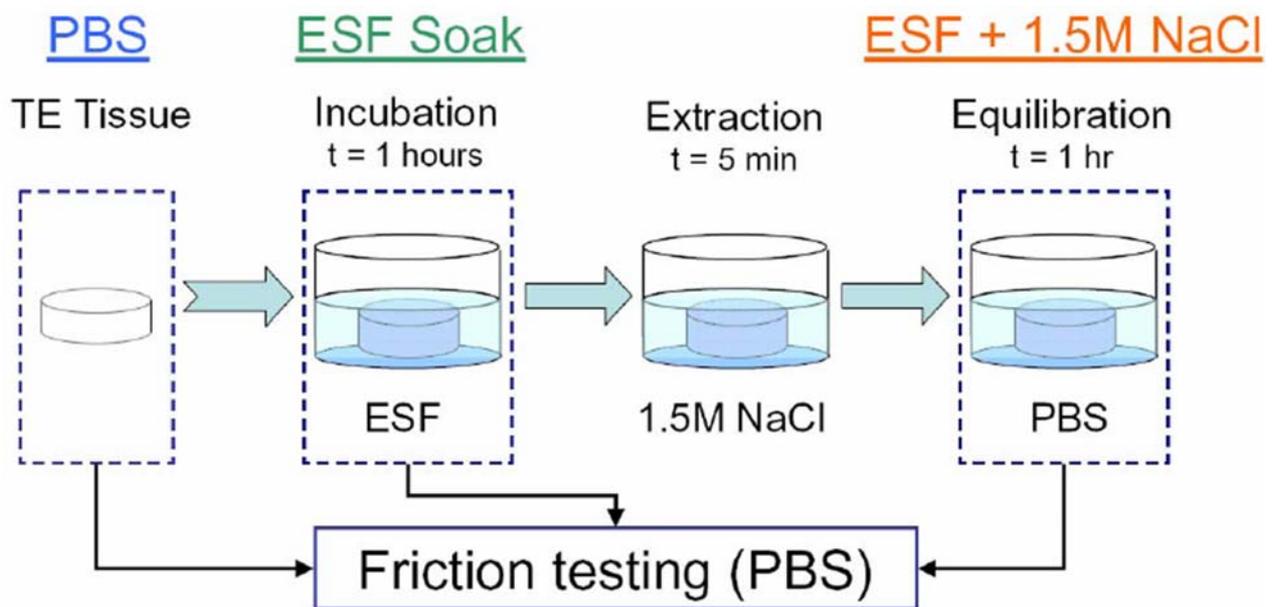
Figure 2: Schematic of steps used to test frictional properties of engineered tissue. Cultured tissues at each time point were tested in PBS (PBS). Following testing, the tissue was incubated for 1 hr in ESF, rinsed, and tested for a second time in PBS (ESF Soak). The tissue was then extracted with 1.5M NaCl for 5 minutes and then equilibrated in PBS. Following an hour equilibration, the tissue was tested for a third time (ESF + 1.5M NaCl).

A friction testing protocol (Figure 2) was developed to determine the frictional properties of the engineered cartilaginous tissues with endogenous or exogenous lubricin localization. An initial friction test was run (PBS) to determine μ_{eq} for the thawed samples (n=5 samples/time point/cell type, n=5 acellular controls). After testing, each sample was then incubated in ESF at 20°C for 1 hour and rinsed with PBS, to remove any excess unbound synovial fluid constituents, then friction tested in PBS (ESF Soak). Following the second friction test, the samples were incubated in 1.5M NaCl (Sigma, St. Louis, MO) in PBS at 4°C for 5 min and then equilibrated in PBS at 20°C for 1 hour. Exposure to 1.5M NaCl has been shown to extract lubricin from the surface of cartilage with minimal proteoglycan loss (Jones *et al.*, 2007). Friction coefficients were then measured for a third time (ESF + 1.5M Extract).

Statistical Analysis

All data are presented as mean +/- standard deviation. The effect of treatment on normalized GAG and hydroxyproline content, H_A, and k was determined using a two factor analysis of variance (ANOVA) with a Tukey's honestly significant difference (HSD) *post hoc* test to determine the effects of cell-alginate construct type and culture duration. Lubricin concentration in the conditioned media and μ_{eq} values were analyzed with a linear mixed mode ANOVA model. A two factor test was performed on lubricin ELISA data to determine the effects of cell-alginate construct type and the repeated measure of culture duration. A three factor test was performed on μ_{eq} data to determine the effects of cell-alginate construct type, culture duration, and the repeated measure of lubrication treatment. All statistical analysis was carried out using SPSS (SPSS Inc, Chicago, IL) with calculated p values being considered significant for p<0.05.

Results

Characterization of engineered tissues

Biochemical analysis of all engineered tissues showed an increase in GAG (Figure 3a) and hydroxyproline (Figure 3b) content, corresponding to localization of ECM molecules within the constructs over culture time. GAG content (Figure 3a) was similar in CON (5.76 +/- 0.40 µg GAG/mg tissue w/w) and MEN (5.01 +/- 0.29 µg GAG/mg tissue w/w) seeded alginate constructs; however over six weeks in culture, MSC seeded constructs only reached approximately 25 to 30% of GAG accumulation (1.60 +/- 0.16 µg GAG/mg tissue w/w) achieved by the other engineered tissue. MSC generated tissue increased in GAG content over 2 and 4 weeks (p<0.03) but had lower GAG content than CON and MEN (p<0.001) at all time points. Hydroxyproline content (Figure 3b), an indicator of collagen content, similarly increased in all constructs at all points in culture (CON = p<0.001, MEN = p<0.001, MSC = p<0.03). Throughout culture CON consistently localized more hydroxyproline than MSC (p<0.004) but less than MEN (p<0.001) with MEN localizing 2.5 times more (3.34 +/- 0.17 µg hydroxyproline/mg w/w) than CON (1.42 +/- 0.07 µg hydroxyproline/mg w/w) and 6.5 times that of MSC constructs (0.55 +/- 0.11 µg hydroxyproline/mg tissue w/w) at six weeks.

Confined compression testing revealed differences in equilibrium moduli and permeability between cell types over the time in culture (Figure 3c). H_A for CON (31 kPa) and MEN (40 kPa) seeded alginate disks similarly increased by three to four times over six weeks in culture (each, p<0.001) with MEN possessing a higher H_A than CON after 2 weeks (p<0.025). Additionally, MEN and CON disks had a higher H_A than both MSC and acellular control disks after 2 weeks (p<0.001). MSC seeded
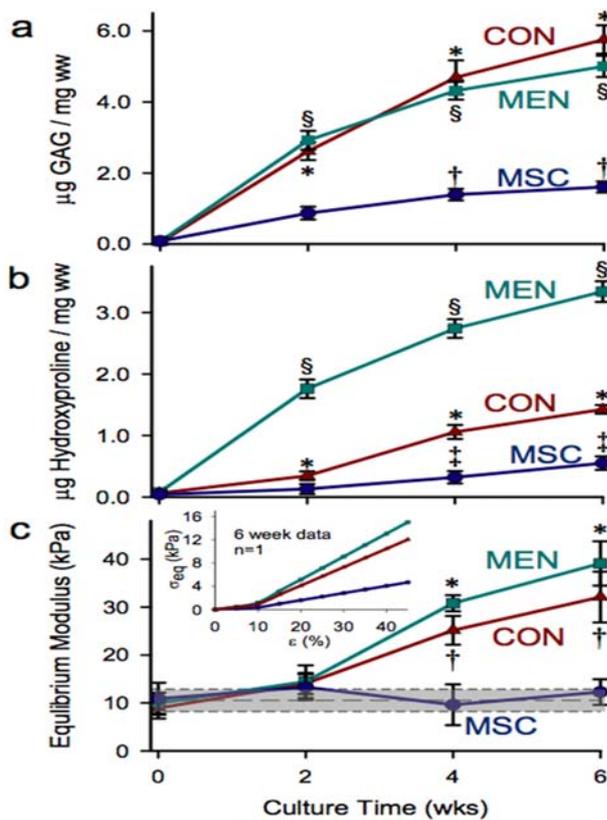
Figure 3: Characterization of biochemical and mechanical properties of the engineered cartilaginous tissues including: (**a**) GAG content normalized to tissue wet weight († = $p<0.001$ for MSC compared to all others, * = $p<0.05$ for CON compared to MEN), (**b**) hydroxyproline content normalized to tissue wet weight (‡ = $p<0.05$ for CON compared to all others), and (**c**) equilibrium modulus († = $p<0.001$ for MSC vs. CON and MEN, * = $p<0.05$ for MEN vs CON). Overlaid gray box represents mean +/- SD of acellular controls ($p<0.001$ for controls compared to CON and MEN after 2 weeks). All data are presented as mean +/- SD with n=5. Inset: Representative equilibrium stress (μ_{eq}) – strain (μ) curves for 6 week samples.

Figure 4: Cumulative release of lubricin to the media over six weeks in culture († = $p<0.02$ for MSC vs. all others, * = $p<0.05$ for CON vs MEN). Data is presented as mean +/- SD with n=6.

Lubricin IHC staining revealed no reactivity to lubricin for any cell-alginate construct at zero weeks, as illustrated by CON samples (Figure 5a – 3A4 column). Minimal immunoreactivity was observed at two weeks (data not shown) and six weeks (Figure 5b – 3A4 column), with some focal staining noted, particularly on the surface of meniscal samples (Figure 5b – 3A4 column, bottom). However, it may be noted that comparisons of lubricin release and localization between the constructs could, at least in part, reflect potential differences in antibody immunoreactivity between species. Incubation with BSF preceding IHC staining showed no immunoreactivity to lubricin at zero weeks for any tissue, CON samples shown (Figure 5a – BSF+3A4 column). However, significant immunoreactivity was noticed in four week (data not shown) and six week samples (Figure 5b – BSF+3A4 column), particularly at the surface of CON seeded samples (Figure 5b – BSF+3A4 column, middle) and throughout the bulk of MEN seeded samples (Figure 5b – BSF+3A4 column, bottom).

Friction testing
Friction testing revealed differences in μ_{eq} with cell type, culture duration, and synovial fluid exposure (Figure 6). For all cell types over all culture times μ_{eq} was similar (~0.45) for engineered constructs and acellular controls (0.458 +/- 0.021). Incubation of the engineered tissues in ESF had no effect on zero week samples, however CON and MEN samples produced lower μ_{eq} over culture duration (μ_{eqCON} = 0.231 +/- 0.022, μ_{eqMEN} = 0.218 +/- 0.027) compared to MSC (μ_{eqMSC} = 0.372 +/- 0.009, $p<0.001$). The friction coefficient of ESF-soaked MSC constructs decreased by 10% at four weeks ($p<0.03$) and 20% at six weeks ($p<0.001$) (Figure 6a) while μ_{eq} of ESF-soaked CON (Figure 6b) and MEN (Figure 6c) samples decreased by 20% at 2 weeks and 50% at four and six weeks ($p<0.001$). Following the extraction protocol with 1.5M NaCl in PBS, μ_{eq} for all engineered tissues at 2, 4, and 6 weeks increased and returned to values similar to those obtained prior to ESF incubation. No statistical difference is noted between ESF + 1.5M Extract samples and either PBS or acellular controls for all cell types and culture times tested.

constructs showed no change in equilibrium modulus over six weeks in culture (11 kPa) or compared to acellular controls. Permeability decreased over culture (data not shown) from an initial value of 5.5×10^{-12} m^2 Pa^{-1} s^{-1} to 1.3×10^{-12} ($p<0.001$), 1.0×10^{-12} ($p<0.001$), and 3.8×10^{-12} m^2 Pa^{-1} s^{-1} ($p<0.05$) for CON, MEN, and MSC respectively.

Analysis of lubricin synthesis and localization
Analysis of culture medium (Figure 4) showed production and loss of lubricin to the media by all cell-alginate constructs over time. MSC constructs released approximately ten-fold more lubricin (25 mg) to media at 42 days compared to CON (2.7 μg) or MEN (0.9 μg) samples. The rate of release of lubricin from MSC seeded constructs was similar to CON and MEN seeded constructs over the first ten days, after which the rate of release from MSC-alginate constructs increased dramatically.
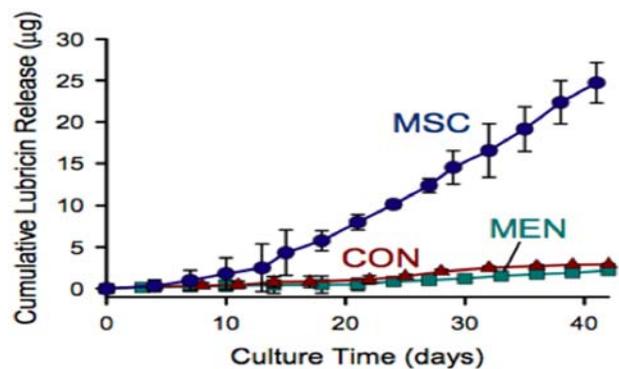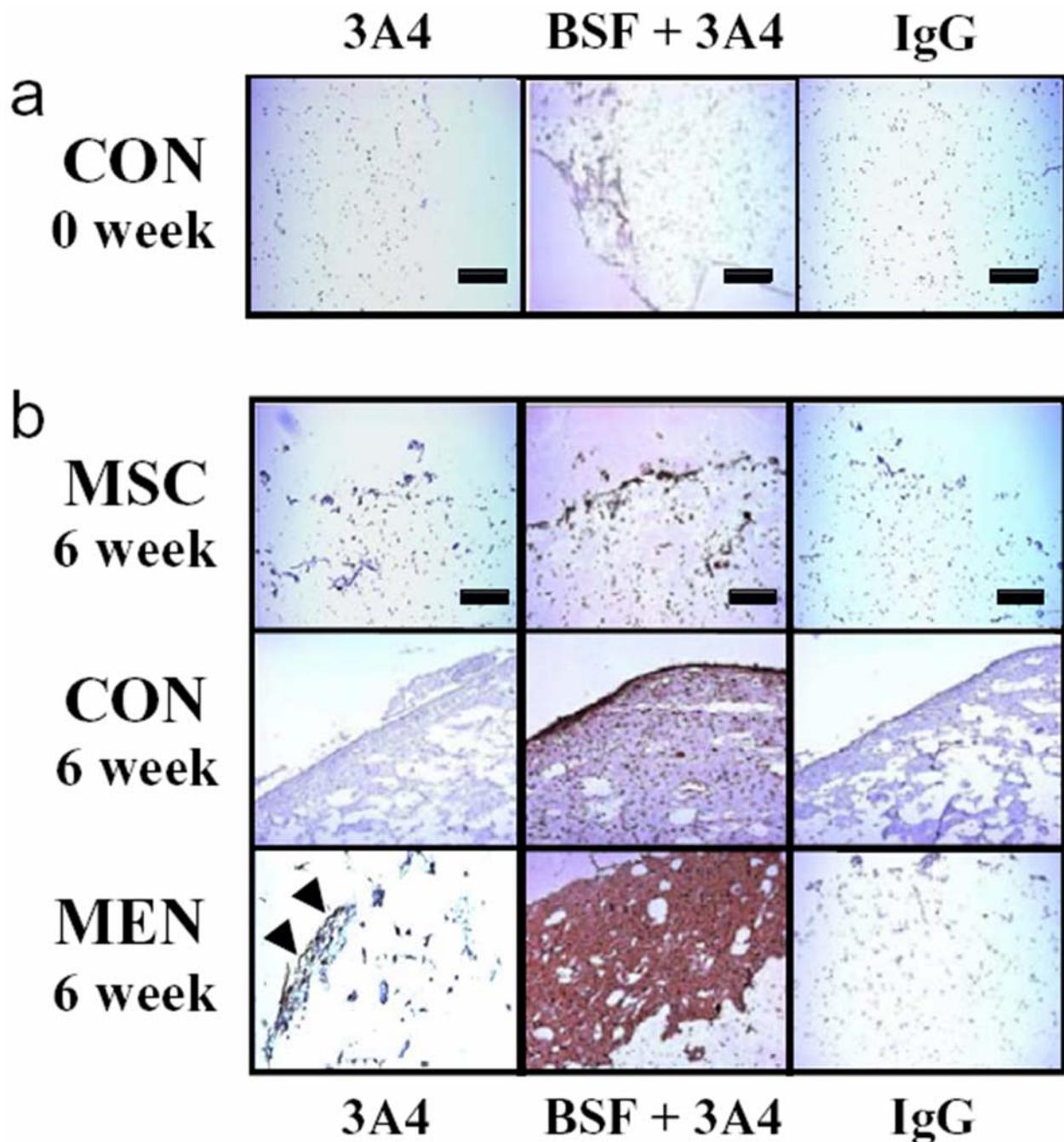
Figure 5: IHC staining of zero week alginate-CON constructs (**a**) and six week alginate-cell tissues (**b**) investigating localization of lubricin from endogenous production of lubricin over culture [3-A-4] and exogenous incubation with BSF [BSF + 3-A-4]. Lubricin noted at surface of MEN constructs (▲) after 6 weeks in culture. (Scale bar = 100 μm).

Discussion

This study demonstrates that articular chondrocytes, meniscal fibrochondrocytes, and chondrogenically differentiated MSCs all produce lubricin in 3D alginate culture. The retention of lubricin in these constructs was limited, with only meniscal constructs showing some focal surface localization of endogenously produced material. The engineered tissues varied in ability to localize lubricin when exposed to BSF, with meniscal constructs retaining material throughout the bulk of the sample, articular cartilage constructs localizing material at the surface, and MSC constructs showing little localization.

The limited localization of endogenous lubricin in engineered constructs was not sufficient to lower friction coefficient. However, the ability to localize lubricin upon BSF exposure appeared to correlate with changes in friction coefficient, with CON and MEN constructs being significantly more lubricated than MSC constructs. Together, these data suggest that the production of matrix that is capable of localizing lubricin may be just as or more important than lubricin production in the lubrication of engineered tissues.

Differences in lubricin release patterns were present based upon cell type, with higher amounts released from MSC cultures, particularly after 12 days. This may
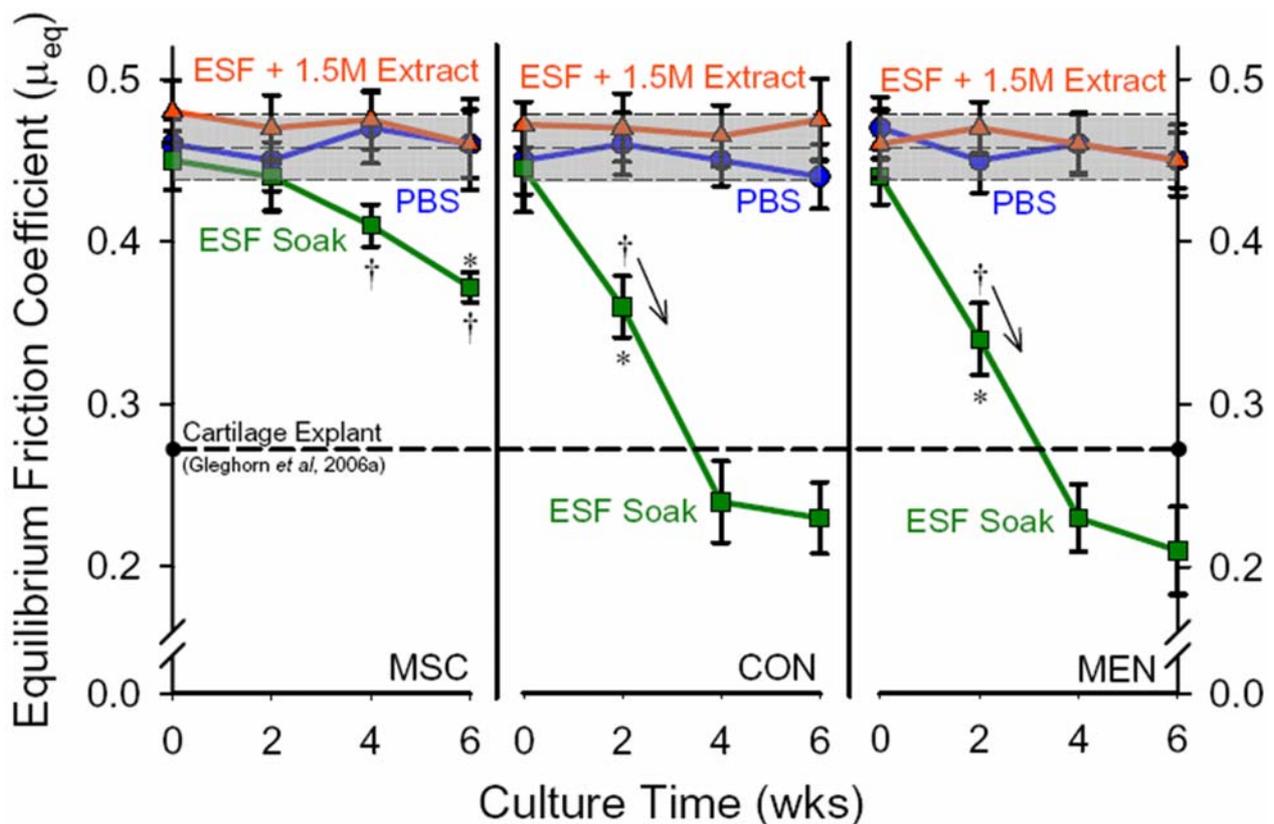
Figure 6: Temporal profile of equilibrium friction coefficient of engineered tissues tested in PBS (PBS), following incubation in ESF (ESF Soak), and following lubricin extraction (ESF + 1.5M NaCl) († = p<0.001 for ESF Soak compared to PBS and ESF + 1.5M NaCl, * = p<0.05 for ESF Soak compared to ESF soak at all other time points for a given cell type). Overlaid grey box represents mean +/- SD of acellular controls (p<0.001 for controls compared to ESF Soak at 2, 4 and 6 weeks for CON and MEN and 4 and 6 weeks for MSC). Reference value (mean) of a cartilage explant tested in the same system (μ_{eq}=0.278 +/- 0.018) is indicated overlaid on the plot (- - -). Data are presented as mean +/- SD with n=5.

represent a distinct phase of chondrogenic differentiation of MSCs. In native cartilage and meniscus, it is well established that only cells from the surface zone express lubricin (Schumacher *et al.*, 1994; Klein *et al.*, 2003; Schumacher *et al.*, 2005). The relatively low release from CON and MEN alginate disks may be due to a limited population of lubricin expressing cells in the seeded constructs, as cells from all areas of the tissue were utilized rather than harvesting cells from superficial tissue areas. The high lubricin release from MSC constructs may be due to a larger population of MSCs differentiating into a lubricin expressing phenotype. Alternatively, media constituents may also play a role in increased lubricin release in MSC seeded constructs. TGF-β was used to promote chondrogenic differentiation of MSCs in this study. However, TGF-β has also been implicated in upregulation of lubricin biosynthesis in cartilage explant cultures (Flannery *et al.*, 1999), and as such, likely contributed to the high level of lubricin release by MSCs seen here.

This study utilized three common cell sources for engineering cartilaginous tissue resulting in engineered tissue with similar ECM composition and mechanical properties to previous studies (Chang *et al.*, 2001; Mauck

et al., 2002) of these engineered tissues. Over all culture times, MSC-, CON- and MEN-seeded alginate constructs had the same frictional properties as unseeded alginate disks. The inherent boundary lubricated equilibrium friction coefficient of the engineered tissues ($\mu_{eq} \sim 0.45$) is greater than that reported in preliminary studies of patellofemoral groove cartilage explants tested under the same conditions with μ_{eq}=0.278 +/- 0.018 and the same cartilage following lubricin extraction with 1.5M NaCl having a μ_{eq}=0.347 +/- 0.022 (Gleghorn *et al.*, 2006a). While no change in lubrication was evident, MSCs, CON, and MEN in alginate all produced lubricin in static culture as evidenced by lubricin release to media. Immunohistochemical analysis revealed low levels of lubricin at the surface of engineered tissue for six week cultured tissues even though incubation with BSF demonstrates the ability of lubricin to significantly bind to the tissue. Taken together these data suggest insufficient localization of lubricin at the surface of these engineered tissues while in culture.

IHC revealed the absence of lubricin localized on zero week alginate constructs after incubation with BSF. These data suggests that localization of lubricin on the surfaces of joint tissues such as articular cartilage (Schumacher *et*

al., 1994; Klein *et al.*, 2003) and meniscus (Schumacher *et al.*, 2005) may not be a simple adsorption mechanism. Together, these data suggest that controlling localization of lubricin in engineered tissues may be critical for proper lubricating function.

Constructs from all cell types were lubricated by ESF at later culture times with CON and MEN disks able to achieve a μ_{eq} similar to that of a cartilage explant after 4 weeks. Further lubrication was reversed with 1.5M NaCl, a protocol known to extract lubricin from the surface of articular cartilage (Jones *et al.*, 2007). This reversible lubrication in tandem with IHC data showing an increase in lubricin localization over culture time with incubation in ESF suggests production of ECM components capable of localizing lubricin. The ability to localize lubricin seen by a decrease in μ_{eq} is cell type dependant with CON and MEN similar but greater than MSC generated tissues. The localization of lubricin at the tissue surface plays a role in cartilage tissue lubrication.

The mechanism of boundary lubrication of cartilaginous tissues has not been fully elucidated. This study demonstrates that molecular modification of the tissue surface by localization and subsequent removal of lubricin altered the frictional properties of the tissue. Further investigations are needed to identify which ECM molecules localize lubricin at the tissue surface. Understanding the mechanical implications of the surface localization of lubricin is an important consideration for creating functional tissue engineered cartilage with appropriate low friction, low wear properties.

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Discussion with Reviewers

R. Sah: For friction coefficients, were the decreases significant with time for a given treatment? Were the differences significant between construct groups? Was there an interactive effect of time and cell type on friction? **Authors:** Friction testing revealed differences in μ_{eq} with cell type, culture duration, and friction treatment. No difference was noted between PBS, ESF + 1.5M NaCl, and acellular controls for any cell type over all culture times. A difference was noted between ESF Soak and other friction treatments which was dependant on culture time and cell type. The results, figure 6, and figure 6 caption

have been updated to include indications of statistical difference, within and between groups, including p-values.

R. Sah: An EDTA-free protease inhibitor cocktail was used; the rationale for this is not clear, since proteases inhibited by EDTA are commonly present in cartilage.
Authors: While EDTA does inhibit MMPs commonly found in cartilage, EDTA will also chelate the Ca^{2+} that is utilized to crosslink the alginate gel. Chelation will result in breakdown of the cell-alginate construct and thus alterations in the mechanical properties of the engineered tissue constructs. The EDTA-free protease inhibitor cocktail is a commercial product focused on inhibiting serine and cysteine proteases. MMP protease activity was limited by minimizing the time from thawing to mechanical testing. Samples were thawed, equilibrated in PBS, and mechanically tested within 1 hour.

R. Sah: Exposure to 1.5M NaCl is also likely to extract PG as well as lubricin. How much PG was depleted?
Authors: While PG loss was not measured for these samples, the 1.5M NaCl lubricin extraction protocol described in Jones *et al.* (2007) showed minimal PG loss with a 20 minute extraction of cartilage explants. Our protocol of lubricin extraction for 5 minutes would further minimize PG extraction while extracting lubricin.

M. Wimmer: Is it possible that with MSC cells just more lubricin is found because it cannot bind to matrix molecules and therefore dilutes into the media?
Authors: The authors agree that this is a possibility. However minimal localization of lubricin was seen in CON and MEN seeded tissues throughout culture, so the significant differences in lubricin loss to the media between MSC and other cell seeded constructs is consistent with an overall larger synthesis of lubricin.

M. Wimmer: In the boundary lubrication mode roughness features of the contacting surfaces become an important factor for friction. What were the roughness values of the glass plate and the engineered tissue constructs? Where those comparable?
Authors: The root mean squared (RMS) roughness of the glass was measured to be 5nm +/- 0.17nm using a MicroXAM non-contact 3D optical profilometer (ADE Phase Shift, Tucson AZ). The surface characteristics of the engineered constructs were not characterized, however the RMS roughness of an acellular alginate gel, created utilizing the same methods described in these studies is 2.4µm +/- 0.16µm. The difference in roughness between the alginate gel and the glass is on the same order of magnitude as a patellofemoral groove cartilage explant with a RMS roughness of 1.5µm +/- 0.08µm. Since the difference in surface roughness is so large between the alginate and the glass counterface, we would expect any effects on the boundary mode μ_{eq} to be dominated by the surface characteristics of the alginate/tissue.

M. Wimmer: How many cycles did a typical friction test last? How wear resistant were the TE constructs? Did they suffer any damage?
Authors: The translation between the sample and the glass counterface was 5cm in each direction, travelling at 0.32mm/sec. These parameters result in approximately 23 cycles. Following friction testing there were no quantitative measurements performed to characterize wear or surface damage. Gross inspection of the samples revealed minimal apparent differences to the articulated surface or overall tissue geometry/structure compared to surfaces before friction testing.

M. Wimmer: A coefficient of friction of 0.45 is quite high. How quickly was equilibrium reached? (in cartilage experiments friction typically starts low and then rises with time).
Authors: The temporal friction profile for the engineered constructs and acellular gels is qualitatively similar to those seen in cartilage explants (Krishnan *et al.*, 2004) [i.e. friction coefficient (μ) was lowest upon instantaneous application of normal strain (μ_{min}) and highest as $t \rightarrow \infty$ (μ_{eq})]. To ensure complete relaxation (and therefore minimize the effect of pressurization on μ), a poroelastic model of material behaviour was used to calculate μ_{eq}. In this system, mechanical relaxation time constants for six week constructs were on the order of 700 seconds as compared to cartilage explants which are on the order of 1200 seconds. Additionally, as a result of the engineered constructs having significantly higher permeability and thus an inability to pressurize as well as native cartilage, μ_{min} is significantly higher for the engineered constructs ($\mu_{min} \sim 0.092$) compared to cartilage explants ($\mu_{min} \sim 0.048$) tested under the same loading conditions.